Small Molecule Therapeutics

The CDK4/6 Inhibitor LY2835219 Overcomes Vemurafenib Resistance Resulting from MAPK Reactivation and Cyclin D1 Upregulation

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Abstract

B-RAF selective inhibitors, including vemurafenib, were recently developed as effective therapies for melanoma patients with B-RAF V600E mutation. However, most patients treated with vemurafenib eventually develop resistance largely due to reactivation of MAPK signaling. Inhibitors of MAPK signaling, including MEK1/2 inhibitor trametinib, failed to show significant clinical benefit in patients with acquired resistance to vemurafenib. Here, we describe that cell lines with acquired resistance to vemurafenib show reactivation of MAPK signaling and upregulation of cyclin D1 and are sensitive to inhibition of LY2835219, a selective inhibitor of cyclin-dependent kinase (CDK) 4/6. LY2835219 was demonstrated to inhibit growth of melanoma A375 tumor xenografts and delay tumor recurrence in combination with vemurafenib. Furthermore, we developed an in vivo vemurafenib-resistant model by continuous administration of vemurafenib in A375 xenografts. Consistently, we found that MAPK is reactivated and cyclin D1 is elevated in vemurafenib-resistant tumors, as well as in the resistant cell lines derived from these tumors. Importantly, LY2835219 exhibited tumor growth regression in a vemurafenib-resistant model. Mechanistic analysis revealed that LY2835219 induced apoptotic cell death in a concentration-dependent manner in vemurafenib-resistant cells whereas it primarily mediated cell-cycle G1 arrest in the parental cells. Similarly, RNAi-mediated knockdown of cyclin D1 induced significantly higher rate of apoptosis in the resistant cells than in parental cells, suggesting that elevated cyclin D1 activity is important for the survival of vemurafenib-resistant cells. Altogether, we propose that targeting cyclin D1–CDK4/6 signaling by LY2835219 is an effective strategy to overcome MAPK-mediated resistance to B-RAF inhibitors in B-RAF V600E melanoma. Mol Cancer Ther; 13(10); 2253–63. ©2014 AACR.

Introduction

B-RAF is the most commonly mutated driver oncogene in melanoma, with activating mutations in codon 600 occurring in almost 50% of the patients (1, 2). Treatment with B-RAF selective inhibitors, such as vemurafenib or dabrafenib, has demonstrated significant benefit in melanoma patients with B-RAF V600E mutation, with extended patient progression-free survival and median overall survival compared with chemotherapy (3–6). However, these responses were relatively short-lived, and drug resistance generally developed within 5 to 7 months (4, 6). Thus, the emergence of resistance remains a considerable therapeutic challenge to achieve durable responses and prolonged survival in these patients.

A variety of molecular mechanisms are identified to be involved in resistance to B-RAF inhibition. The most common resistant mechanism is MAPK pathway reactivation, which is caused by genetic mutation of MEK or different Ras isoforms (7–9), upstream activation of receptor tyrosine kinases (RTK) such as FGFR3 and c-Met (10, 11), expression of B-RAF V600E splice variants that dimerize in presence of the B-RAF inhibitor (12), amplification of B-RAF (13, 14), and upregulation of MAP3Ks such as COT or C-RAF (15, 16). Alternatively, activation of MAPK-redundant pathways such as PI3K/Akt as a consequence of PTEN loss (17) or overexpression of RTKs such as PDGFRβ and IGF1R have also been reported to induce resistance in B-RAF V600E melanoma (7, 18, 19). In addition, secretion of growth factors such as HGF or FGF has also been implicated in resistance to B-RAF inhibition (10, 11, 20, 21). Although the resistant mechanisms are frequently associated with MAPK reactivation, treatment with the MEK inhibitor trametinib, or with trametinib plus dabrafenib, has not been very effective in patients.
who have previously failed B-RAF inhibitor (22), suggesting that subsequent targeting of MAPK signaling alone is not sufficient. Therefore, despite recent advances in the clinic, drug resistance upon selective B-RAF inhibition remains a considerable therapeutic challenge in clinic.

Constitutive activation of cyclin-dependent kinases (CDK) and deregulation of cell cycle are common features across several cancer types, including melanoma. P16INK4a, a tumor suppressor gene and a negative regulator of CDK4, is deleted in 38% of melanoma (2, 23). In addition, germline mutations and amplification of CDK4 gene have been identified in melanoma, which leads to unrestricted CDK4 activity and increased cell proliferation (2, 24). In general, regulation of cell-cycle entry in proliferating adult mammalian cells is controlled by D-cyclins which bind and activate CDK4 and CDK6 to promote phosphorylation of retinoblastoma (Rb) protein and G1 to S transition (25). The RAS–MAPK pathway is known to control cell-cycle entry via upregulation of cyclin D1 in several cell types (26, 27). Inhibition of MAPK signaling by B-RAF inhibitors decreases cyclin D1 expression and upregulates CDK inhibitor p27kip1 levels, thus blocking cell-cycle entry in B-RAF V600E melanoma (26, 27). Overexpression of cyclin D1 is linked to resistance to B-RAF inhibition (28). CCND1 is amplified in 11% of melanoma, including 17% of B-RAF V600E melanoma, thus suggesting a potential role of cyclin D1 in intrinsic resistance to B-RAF inhibitors (2, 28). However, the role of cyclin D1 in acquired resistance to vemurafenib has not been described, and the therapeutic value of targeting cyclin D1/Rb axis to overcome vemurafenib resistance has not been explored.

In this study, we have generated multiple in vitro cell lines and an in vivo model of resistance to vemurafenib and discovered that MAPK reactivation and cyclin D1 elevation are common in these resistant models. We describe that cyclin D1 is an important mediator of vemurafenib resistance and provides a potential therapeutic target to overcome resistance to B-RAF inhibition in B-RAF V600E melanoma. Using these in vitro and in vivo models, we show that cyclin D1 is generally elevated and functions as a critical node for the survival of vemurafenib-resistant cells. We further demonstrate that LY2835219, a selective dual CDK4/6 inhibitor currently in phase II clinic studies (29), can overcome vemurafenib resistance in these resistant models. Altogether, this study sheds new light on mechanisms of resistance to B-RAF inhibition, identifies cyclin D1 elevation concurrent with MAPK reactivation as a common resistant mechanism, and proposes targeting cyclin D1 through CDK4/6 inhibition by LY2835219 as an effective therapeutic strategy to overcome B-RAF resistance.

Materials and Methods

Cell culture, reagents, and transfections

A375, SH4, and A2058 cells were obtained from ATCC on May 7, 2012, July 2, 2012, and June 27, 2006, respectively. M14 cells were purchased from NCI on March 10, 2005. Cells were stored within a central cell bank that performs cell line characterizations. All these cells were passaged for fewer than 2 months after which time new cultures were initiated from vials of frozen cells.

Generation of vemurafenib-resistant cell lines

A375R1, A375R3, M14R, and SH4R models with acquired resistance to vemurafenib were generated by treating respective parental cells with gradually increasing concentrations of vemurafenib, up to 2 µmol/L, as previously described (10). A375R2 cells were generated by treating A375 cells with a high concentration of vemurafenib (2 µmol/L) for 1 week, followed by culturing with vemurafenib (1 µmol/L) for up to 20 passages. Upon establishment of resistance, the inhibitor was withdrawn and all of the cell lines were maintained in regular media for subsequent passages. For A375R3 cells, the NRas Q61K was stably transfected into A375 cells, and single-cell clone was selected and characterized for this study. All of the models retained resistance to vemurafenib for up to 20 passages (data not shown). In this study, all resistant cell lines with less than 10 passages were used for experiments.

Cell proliferation assay

Cells (1 × 10^3 to 3 × 10^5) were normally plated in 96-well plates (BD Biosciences). Cells were treated the next day for 96 hours, and then assessed for viability using CellTiter Glo (Promega), as per manufacturer’s
instructions and a luminescence plate reader (Victor, Perkin Elmer). GraphPad Prism 4 software was used to generate sigmoidal dose-response curves and calculate the proliferation IC₅₀.

**Caspase-3/7 activity assay**

Cells (5 x 10⁵) were plated in 96 well plates (BD Biosciences). Cells were treated the next day for 24 to 48 hours and then assessed for caspase-3 activity by Caspase-Glo-3/7 Assay (Promega), as per manufacturer’s instructions and a luminescence plate reader (Victor, Perkin Elmer).

**Preparation of cell lysates and immunoblotting**

Cells and tumor tissues were lysed using either RIPA lysis buffer (Bio-Rad; cell lines) or 1% SDS solution (tumor lysates), containing 1× phosphatase and proteinase inhibitor cocktail (Pierce). Tumor lysates were prepared with freshly frozen tumor samples. Approximately 200 mg of tumor tissue was homogenized and lysed using 0.5 mL of lysis buffer. The protein concentration of individual samples was determined with DC Protein Assay Kit (Bio-Rad). SDS-PAGE was performed on cell lysates containing 20 μg of total protein using 4% to 20% Novex tri-glycine gradient gels (Invitrogen). Protein was transferred onto 0.2-μm nitrocellulose membranes using Trans-Blot Turbo Transfer system to 20% Novex tri-glycine gradient gels (Invitrogen). Dislodged cells were collected, resuspended, and plated in DMEM (Thermo Scientific) supplemented with 50% FBS (Invitrogen) and 10% penicillin/streptomycin (Invitrogen). After 1 week, cells were switched to regular media (DMEM + 10% FBS).

**Flow cytometry**

Cell pellets were fixed in 70% ethanol for 30 minutes at −20 °C and then washed with PBS. Fixed cells were stained with propidium iodide (PI)/Triton X-100 staining solution and incubated for 30 minutes at room temperature. Fixed cells were then subjected to flow cytometric analysis on the Beckman Coulter FC 500 Cytomics flow cytometer. Data were analyzed with ModFit LT 3.0 (Verity House Software).

**In vivo experiments and drug administration**

All animal studies were performed in accordance with American Association for Laboratory Animal Care institutional guidelines. The Eli Lilly and Company Animal Care and Use Committee approved all the experimental protocols. Athymic nude female mice were inoculated with 0.2 mL of 1 x 10⁷ A375 cells, prepared in a 1:1 Matrigel to media mixture, in the hind flank region. A total of 60 mice, 8 to 10 in each group, were used for compound treatments and vemurafenib-resistant model development in each study. Vemurafenib was formulated by dissolving in DMSO in a volume equivalent to 5% of the final formulation volume, and then the remaining volume was added to the solution of 1% methylcellulose in distilled water. CDK4/6 inhibitor LY2835219 was formulated in 1% HEC in 20 mmol/L phosphate buffer, pH 2.0. Treatment was administered orally (gavage) with the dose schedules described in each study. Tumor growth and body weight were monitored over time to evaluate efficacy.

**Generation of cell lines from xenograft tumors**

Tumors were aseptically removed from the animals, washed with cold PBS, then minced, and trypsinized in 10-cm petri dish containing 5 mL of TrypLE reagent (Invitrogen) for 15 minutes at 37°C. Dislodged cells were collected, resuspended, and plated in DMEM (Thermo Scientific) supplemented with 50% FBS (Invitrogen) and 10% penicillin/streptomycin (Invitrogen). After 1 week, cells were switched to regular media (DMEM + 10% FBS).

**Results**

**Development of vemurafenib-resistant cell lines**

To study the resistant mechanisms of B-RAF inhibition, we generated several vemurafenib-resistant cells outlined in Table 1. A375R1, A375R3, M14R, and SH4R cells were generated by treating their respective parental cells with gradually increasing concentrations of vemurafenib, up to 2 μmol/L as described previously (10). The resistant mechanism of A375R1, A375R3, and M14R cells was associated with RTK/RAS activation and MAPK reactivation (10). MAPK reactivation was also observed in SH4 cells, but the underlying mechanism has not been fully characterized. A375R2 cells were generated by treating A375 cells with a constant 2 μmol/L vemurafenib as described (12). Consistent with the earlier reports, the resistance of A375R2 cells to vemurafenib is conferred by expression of B-RAF splice variant (Supplementary Fig. S1A). The A375R4 cells were generated through stable transfection of NRas Q61K mutant and single clone selection, which resulted in vemurafenib resistance (Supplementary Fig. S1B).

**MAPK reactivation and cyclin D1 elevation in vemurafenib-resistant cells and their sensitivity to CDK4/6 inhibitor LY2835219**

As shown in Table 1, all in vitro generated resistant cell lines, A375R1-R4, M14R, and SH4R, demonstrated resistance to vemurafenib. Importantly, these resistant cells showed enhanced MAPK activation and cyclin D1 elevation (Fig. 1). MAPK pathway activation and loss of cell-cycle control are generally the hallmarks of melanoma (2). Therefore, we used a selective CDK4/6 dual inhibitor LY2835219 and evaluated its growth-inhibitory effects in a panel of melanoma cell lines that are either sensitive or resistant to vemurafenib mediated by diverse mechanisms (Table 1). Interestingly, B-RAF V600E melanoma cells that are either sensitive to vemurafenib, such as A375, M14, and SH4, or resistant to vemurafenib, such as A375R1-4, M14R, and SH4R, demonstrated comparable sensitivity to LY2835219 with IC₅₀ values ranging from 0.3 to 0.6 μmol/L (Table 1). On the contrary, B-RAF V600E mutant A2038 cells with de novo resistance to vemurafenib via MAPK-independent
mechanism (i.e., PTEN deletion) were relatively insensitive to LY2835219 (Table 1).

Antitumor effects of vemurafenib, LY2835219, and their combination in A375 xenograft model

In vitro analysis revealed that parental B-RAF V600E melanoma cells, such as A375 cells, were sensitive to both vemurafenib and LY2835219. To compare their activities in vivo, we tested their antitumor effects as single agents or in combination in an A375 xenograft model. As demonstrated in Fig. 2A, vemurafenib treatment at 15 mg/kg twice daily induced significant tumor growth regression in the first 2 weeks of treatment. Tumor growth was also inhibited by LY2835219 in a dose-dependent fashion (Fig. 2B). Statistically significant tumor growth inhibition by LY2835219 was observed at 45 or 90 mg/kg once daily dose schedule. Furthermore, analysis of tumor lysates showed that LY2835219 treatment significantly reduced pS780-Rb and pS10-Histone H3 levels, indicating inhibition of cell cycle and a decrease in proliferating tumor cells as a result of CDK4/6 inhibition (Fig. 2C). When xenograft tumors were treated with a combination of vemurafenib and LY2835219, an additive antitumor growth effect was observed (Fig. 2D).

Development of in vivo vemurafenib-resistant model and efficacy of CDK4/6 inhibitor LY2835219 in this resistant model

To evaluate the efficacy of LY2835219 in the vemurafenib-resistant tumors, we developed an in vivo vemurafenib-resistant model as shown in Fig. 3A. Following the establishment of tumors, vemurafenib at 15 mg/kg was administered orally twice a day. Consistent with the previous observations in this model, vemurafenib-treated mice demonstrated significant regression in tumor volume initially. We continued dosing with vemurafenib until resistance was evident. Approximately 40 to 45 days after dosing, many animals relapsed and resistant tumors started to emerge. When vemurafenib-resistant tumors reached sizes approximately 600 to 1,000 mg, the animals were randomized into 2 groups, each 7 to 8 animals. One group continued to be dosed with vemurafenib, and the other group was switched to CDK4/6 inhibitor LY2835219 treatment alone at 90 mg/kg once daily schedule. As demonstrated in Fig. 3A, LY2835219-treated mice demonstrated significant tumor growth regression, whereas the tumors in vemurafenib-treated mice continued to grow. Furthermore, LY2835219-mediated tumor growth inhibition was maintained upon cessation of the treatment. To rule out the possibility that the tumors had developed a dependence on vemurafenib for continued growth as previously described (14), we repeated the

![Figure 1. MAPK reactivation and cyclin D1 elevation in vemurafenib-resistant melanoma cell lines.](image-url)

Table 1. Overview of drug sensitivities, mutational status, and mechanisms of resistance of melanoma cell lines tested in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mutation</th>
<th>Sensitivity to vemurafenib</th>
<th>Resistance mechanism</th>
<th>Vemurafenib IC&lt;sub&gt;50&lt;/sub&gt;, nmol/L</th>
<th>LY2835219 IC&lt;sub&gt;50&lt;/sub&gt;, nmol/L</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Average SD</td>
<td>Average SD</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>A375</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Sensitive</td>
<td>—</td>
<td>125 35</td>
<td>412 103</td>
</tr>
<tr>
<td>A375-R1</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>FGF-mediated ERK activation</td>
<td>4,541 939</td>
<td>407 54</td>
</tr>
<tr>
<td>A375-R2</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>p61–B-RAF splicing</td>
<td>3,364 1,449</td>
<td>361 149</td>
</tr>
<tr>
<td>A375-R3</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>FGF-mediated ERK activation</td>
<td>3,035 1,781</td>
<td>555 132</td>
</tr>
<tr>
<td>A375-R4</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>NRas Q61K</td>
<td>8,214 2,640</td>
<td>468 82</td>
</tr>
<tr>
<td>M14</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Sensitive</td>
<td>—</td>
<td>303 97</td>
<td>843 224</td>
</tr>
<tr>
<td>M14-R</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>FGF-mediated ERK activation</td>
<td>5,337 1,190</td>
<td>538 171</td>
</tr>
<tr>
<td>SH4</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Sensitive</td>
<td>—</td>
<td>489 131</td>
<td>379 45</td>
</tr>
<tr>
<td>SH4-R</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>Not characterized</td>
<td>7,775 827</td>
<td>374 33</td>
</tr>
<tr>
<td>A2058</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>PTEN inactivation</td>
<td>3,586 115</td>
<td>1,729 237</td>
</tr>
<tr>
<td>A375-RV1</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>p61–B-RAF splicing</td>
<td>4,413 1,808</td>
<td>221 67</td>
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<tr>
<td>A375-RV2</td>
<td>B-RAF&lt;sup&gt;V600E&lt;/sup&gt;</td>
<td>Resistant</td>
<td>Not characterized</td>
<td>3,619 2,553</td>
<td>368 188</td>
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Figure 2. Antitumor activity of vemurafenib and LY2835219 in A375 xenograft model. A, antitumor activity of vemurafenib in A375 xenografts. Mice bearing subcutaneous A375 tumors were dosed with vehicle (n = 8) or 15 mg/kg vemurafenib (n = 10) twice daily. The y-axis is mean tumor volume ± SEM. B, antitumor activity of CDK4/6 inhibitor LY2835219 in A375 xenograft model. Mice bearing subcutaneous A375 tumors were dosed with vehicle (n = 7), 22.5 mg/kg (n = 5), 45 mg/kg (n = 5), or 90 mg/kg (n = 5) LY2835219 once daily for 21 days. C, dose-dependent reduction of CDK4/6 activity in A375 xenograft tumors by LY2835219. Mice bearing subcutaneous A375 tumors were dosed with vehicle, 22.5, 45, or 90 mg/kg of LY2835219 once daily. Tumors were collected 24 hours after the third dose. Tumor lysates were prepared and analyzed by immunoblotting using indicated antibodies. D, activities of LY2835219, vemurafenib, and their combination in A375 xenograft model. Mice bearing subcutaneous A375 tumors were dosed for 21 days with either vehicle (n = 8), 45 mg/kg LY2835219 (n = 8), 10 mg/kg vemurafenib (n = 8), or combination of 45 mg/kg LY2835219 and 10 mg/kg vemurafenib (n = 8). LY2835219 was dosed once daily and vemurafenib was dosed twice daily. The brown line below the x-axis indicates the dosing (Rx) period in all studies. The y-axis is mean tumor volume ± SEM. The pairwise comparisons of each treatment versus control are all statistically significant (P < 0.001). The tests between the combination group and each single agent group are also statistically significant (P < 0.001). The P value for the 2-way interaction to determine whether combinations are different from additive is not statistically significant (P = 0.657), thus indicating that the combination of these 2 agents is additive rather than synergistic.
same experiment with a vehicle control arm in addition to the continued vemurafenib arm and the LY2835219 treatment group. In this study, both the vehicle control group and the vemurafenib group showed indistinguishable and continued growth after vemurafenib withdrawal (Supplementary Fig. S2). These results suggest that CDK4/6 inhibitor LY2835219 as a single agent is effective in overcoming vemurafenib resistance in this in vivo model. We further investigated the molecular mechanism behind increased sensitivity of resistant tumors to vemurafenib. We found hyperelevated levels of phospho-ERK, phospho-MEK, cyclin D1, and phospho-Rb (S780, S807, S811) in these vemurafenib-resistant tumors, indicating upregulation of MAPK and CDK activity (Fig. 3B). This observation is consistent with previous findings that upregulation of cyclin D1 was associated with enhanced sensitivity of cancer cells to CDK4/6 inhibitors (30, 31). Interestingly, analysis of whole-exome sequencing data derived from parental A375 and vemurafenib-resistant A375R1 cells revealed a copy number gain of 11q13 region in the A375R1 cells and amplification of genes in the 11q13 locus including CCND1, FG3, FG4, and FG19 (Supplementary Table S1). Copy number variations of CCND1 are currently being evaluated in other resistant cell lines. Taken together, our results indicate that MAPK reactivation and upregulation of cyclin D1 are associated with vemurafenib resistance and sensitivity to CDK4/6 inhibition.

Cyclin D1 upregulation and vemurafenib resistance in cells generated from resistant xenograft tumors

To further understand the mechanism of vemurafenib resistance and increased sensitivity to LY2835219, we generated 2 cell lines from vemurafenib-resistant tumors, hereafter referred to as A375RV1 and A375RV2 cells. Genotype analysis [short tandem repeat (STR)] confirmed that A375RV1 and A375RV2 have the same genotypes of parental A375 cells. Compared with A375 cell line (vemurafenib IC50: 102 nmol/L), A375RV1 and A375RV2 cell lines maintained resistance to vemurafenib with IC50 of 1,520 and 1,095 nmol/L, respectively (Fig. 4A and Table 1). We further characterized these cells and found that phospho-MEK and phospho-ERK levels remained high in the resistant cells in the presence of vemurafenib concentrations as high as 3 μmol/L compared with parental A375 cells where
phospho-MEK and p-ERK were diminished at concentrations as low as 500 nmol/L (Fig. 4B and Supplementary Fig. S3). In addition, we also detected expression of p61-B-RAF splice variant in the A375RV1 cells (Fig. 4B), suggesting that one mechanism of resistance to vemurafenib in this cell line is likely due to alterations in B-RAF splicing. Consistent with the observations from the analysis of the tumor lysates, the cell lines derived from the resistant tumors also showed enhanced expression of cyclin D1 (Fig. 4B and Supplementary Fig. S4C).

LY2835219 induces G1 arrest in the parental cells but apoptosis in vemurafenib-resistant cells derived from tumors

We tested whether the vemurafenib-resistant cells derived from tumors were sensitive to LY2835219 in cell culture and found that LY2835219 inhibited the proliferation of the parental A375 and resistant A375RV1 and A375RV2 cells with similar potencies with IC50 values of 395, 260, and 463 nmol/L, respectively (Fig. 5A). This is consistent with the responses of other in vitro vemurafenib-resistant cells. It is well established that inhibition of cyclin D1–CDK4/6 axis, and subsequent inactivation of Rb, arrests proliferating cells in G1 stage of cell cycle. We performed PI staining and FACS analysis of cell-cycle distribution to study the mechanism of antiproliferative effects of LY2835219 in parental and vemurafenib-resistant cells. As expected, 92% of parental A375 cells arrested in the G1 phase upon LY2835219 treatment (Fig. 5B). However, LY2835219 treatment induced cell death in the resistant cells, up to 90% in the A375RV1 and 69% in A375RV2 cells, in 48 hours as indicated by the presence of a subdiploid peak in the cytometry histograms. Similarly, LY2835219 arrested 75% of parental M14 cells in the G1 stage but induced cell death in 70% of vemurafenib-resistant M14R cells (Supplementary Fig. S4A). Thus, antitumor effects of LY2835219 are differentially mediated in parental and resistant cells, although the IC50 values are similar in CellTiter Glo proliferation assay. We further investigated whether LY2835219-induced cell death in the resistant cells is mediated by apoptosis. As demonstrated in Figs. 5C and 6A, LY2835219 treatment significantly elevated caspase-3/7 activity in A375RV1 and A375RV2 cells and cleaved PARP activity in A375RV1 cells in a concentration-dependent fashion. Similarly, LY2835219 induced higher caspase-3/7 activity and PARP cleavage in other vemurafenib-resistant A375R1 and M14R cells in a concentration-dependent manner (Supplementary Fig. S4B), suggesting that LY2835219 induces apoptosis preferably in the vemurafenib-resistant cells.
Upregulation of cyclin D1 is associated with LY2835219-induced apoptosis and important for the survival of vemurafenib-resistant tumor cells

Treatment with LY2835219 caused a concentration-dependent inhibition of phospho-Rb (S780, S807/S811) with similar potencies in parental and resistant cells, indicating inhibition of CDK4/6 activity and inactivation of Rb function. Furthermore, LY2835219 treatment induced a concentration-dependent enhancement of cyclin D1/D2 proteins in A375 cells, indicative of growth arrest in these cells (Fig. 6A). Consistent with the in vivo data, cyclin D1 levels remained high in vemurafenib-resistant tumor cells (Fig. 6A and B and Supplementary Fig. S4C). We explored whether vemurafenib-resistant cells are dependent on cyclin D1 for their survival. We performed shRNA-mediated selective knockdown of cyclin D1 in the parental and vemurafenib-resistant cells and found that the loss of cyclin D1 decreased phospho-Rb levels in both A375 and A375RV1 cells but induced significantly higher levels of cleaved PARP and cleaved caspase-3 fragments in A375RV1 cells (Fig. 6B). Similarly, specific knockdown of cyclin D1 via siRNA also induced PARP cleavage in A375RV1 and other vemurafenib-resistant cells including A375RV2 and A375R1 cells (Supplementary Fig. S4C), indicating that loss of cyclin D1 induces apoptosis in vemurafenib-resistant cells and not in the parental cells. Cyclin D1 knockdown did not affect phospho-ERK levels (Fig. 6B) or levels of other D-type cyclins, such as cyclin D2 (Supplementary Fig. S4C). Thus, we show that vemurafenib-resistant cells are dependent on cyclin D1 for their survival.

Discussion

In this study, we generated multiple in vitro cell lines and an in vivo model resistant to vemurafenib and discovered that MAPK reactivation and cyclin D1 elevation are associated with acquired resistance in these models. We further describe that inhibition of cyclin D1–CDK4/6 signaling by CDK4/6 inhibitor LY2835219 is an effective therapy to overcome resistance. Expression of B-RAF V600E splice variants, RTK/Ras activation, NRas mutation, and B-RAF amplification are the predominant clinical mechanisms of resistance to vemurafenib that have been observed to date, and all of these resistant mechanisms together with B-RAF mutation induce hyperactivation of the MAPK pathway (7, 12, 13). We found that the cells resistant to vemurafenib with hyperactivation of the MAPK pathway have elevated cyclin D1 expression and that they were sensitive to CDK4/6 inhibitor LY2835219. We subsequently developed a vemurafenib-resistant model in vivo and evaluated the antitumor effect of LY2835219 in this model. Consistent with the in vitro findings, phospho-ERK and cyclin D1 were elevated in these resistant tumors (Fig. 3B). More importantly, LY2835219 induced regression of these vemurafenib-resistant tumors (Fig. 3A). These results suggest that CDK4/6 inhibition represents an effective therapeutic strategy to overcome vemurafenib resistance due to MAPK reactivation and associated with cyclin D1 elevation.

To investigate the mechanism of LY2835219 sensitivity and vemurafenib resistance, we generated resistant cells lines from vemurafenib-resistant xenograft tumors. Molecular analysis of the tumor-derived resistant cell
might be a more effective approach than upfront combination of vemurafenib followed by LY2835219 treatment schedule to vemurafenib (Fig. 3A). These results suggest that upfront combination of CDK4/6 and B-RAF inhibitor may be more efficacious than single-agent therapy. This suggests that upstream combination of CDK4/6 and B-RAF inhibitors may be more efficacious than single-agent therapy. However, a more robust tumor growth regression was observed when CDK4/6 inhibitor LY2835219 was used upon CDK4/6 inhibition by LY2835219, suggesting that these resistant cells are more dependent on cyclin D1–CDK4/6 signaling for survival.

Cyclin D1 and CDK4/6 signaling is required for the survival of vemurafenib-resistant B-RAF V600E melanoma cells. A, cell signaling analysis in A375 and A375RV1 cells treated with CDK4/6 inhibitor LY2835219. Cells were treated with indicated concentrations of LY2835219 for 48 hours. Cell lysates were analyzed by immunoblotting using antibodies indicated. B, cyclin D1 knockdown by shRNA induced apoptosis in vemurafenib-resistant cells. Cells were transduced with lentivirus encoding either control or cyclin D1 shRNA. Cell lysates were collected 72 hours postinfection and analyzed by immunoblotting using indicated antibodies. Cell lysates were collected 72 hours posttransfection and analyzed by immunoblotting using indicated antibodies.

Analysis of cyclin D1 amplification in other resistant cell lines revealed hyperactivation of the MAPK pathway and an increase in cyclin D1 expression just as had been observed in the in vitro resistant cells. Similar to the xenograft studies in mice, resistant cells derived from tumors retained resistance to vemurafenib, as well as sensitivity to LY2835219 (Figs. 4A and 5A). We further examined the mechanism of antiproliferative effects of LY2835219 in parental and resistant cells. As suggested, LY2835219 arrested parental cells in the G1 stage (Fig. 5B and Supplementary Fig. S4A), consistent with the role of CDK4/6 function in progression of cells from G1 to S stage of cell cycle (25). However, treatment of resistant cells with LY2835219 induced apoptosis within 24 to 48 hours in a concentration-dependent fashion (Figs. 5B and C and 6A and Supplementary Fig. S4A and S4B). The surprising pro-apoptotic effects of LY2835219 were observed across a variety of vemurafenib-resistant cell lines, including cells derived from resistant xenograft tumors. Thus, vemurafenib-resistant B-RAF V600E melanomas with hyperactivated MAPK signaling and enhanced cyclin D1 expression are prone to apoptosis upon CDK4/6 inhibition by LY2835219, suggesting that these resistant cells are more dependent on cyclin D1–CDK4/6 signaling for survival.

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resistant versus parental cells (Fig. 6B and Supplementary Fig. S4C). However, these data do not entirely exclude the possibility that either off-target effects of LY2835219 or inhibition of Rb-independent pathways by cyclin D1 knockdown might also contribute to the induction of apoptosis observed selectively in vemurafenib-resistant cells. Altogether, we demonstrate that cyclin D1 is important for survival of vemurafenib-resistant cells with hyperactivation of the MAPK pathway and cyclin D1 upregulation and propose LY2835219, a CDK4/6 dual inhibitor, as a potential therapy to overcome such resistance.

Disclosure of Potential Conflicts of Interest
T.F. Burke, L. Huber, E.M. Chan, and R.P. Beckmann have ownership interest (including patents) in Eli Lilly and Co. No potential conflicts of interest were disclosed by the other authors.

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References
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